L-ARGININE POLYMERS INHIBIT THE DEVELOPMENT OF VEIN GRAFT NEOINTIMAL HYPERPLASIA

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Objective: We sought to determine whether L-arginine polymer treatment of vein grafts enhances vascular production of nitric oxide and inhibits the development of neointimal hyperplasia.

Methods: External jugular veins of New Zealand White rabbits (n = 42) were harvested; treated intraluminally for 15 minutes with phosphate-buffered saline solution or L-arginine polymer 5, 7, or 9 at either 10 or 100 µmol/L; and then grafted into the contralateral carotid artery. Rabbits were killed after 28 days, and 5-µm sections of vessels were stained with hematoxylin and scored for intima/media ratio by using computerized morphometric analysis. Separate veins were treated in a similar fashion with biotinylated polymers and phosphate-buffered saline solution to assess for translocation efficiencies. Finally, vein segments pretreated with either phosphate-buffered saline solution or L-arginine polymers were cultured in Dulbecco's modified Eagle's medium containing lipopolysaccharide (100 µg/mL) and interferon γ (200 U/mL) for 48 hours before measuring nitric oxide levels by means of the Griess reaction.

Results: Biotinylated L-arginine polymers demonstrated a dose- and length-dependent uptake into intimal and medial cells of treated vessels. Nitric oxide levels were significantly higher in vein segments treated with 100 µmol/L of L-arginine polymer 9 compared with control segments. Finally, the intima/media ratio also reflected both length- and concentration-dependent inhibition of neointimal hyperplasia.

Intima/media ratio

	PBS	*R5*	*R7*	*R9*
10 µmol/L	0.909 ± 0.072	0.920 ± 0.073	0.861 ± 0.138	0.710 ± 0.122
100 µmol/L		0.924 ± 0.061	0.581 ± 0.089*	0.529 ± 0.093*

PBS, Phosphate-buffered saline solution; *R,* L-arginine polymer.
**P* < .001 versus phosphate-buffered saline solution and L-arginine polymer 5 controls (Bonferroni-corrected value).

Conclusions: Arginine polymers of sufficient length and concentration were effective in increasing nitric oxide levels and reducing neointimal hyperplasia in this vein graft model. (J Thorac Cardiovasc Surg 2001;121:971-80)

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Despite concerted efforts to increase the use of arterial grafts in recent years, saphenous veins remain the most widely used bypass vessels in coronary artery revascularization procedures.[1] Unfortunately, venous conduits develop accelerated atherosclerosis as a result of neointimal hyperplasia. This is partially responsible for the progressive occlusion that limits 15-year patencies to approximately 50%.[2]

Vein graft injury initially results from increased wall stresses incurred by arterial pressures, transient is-

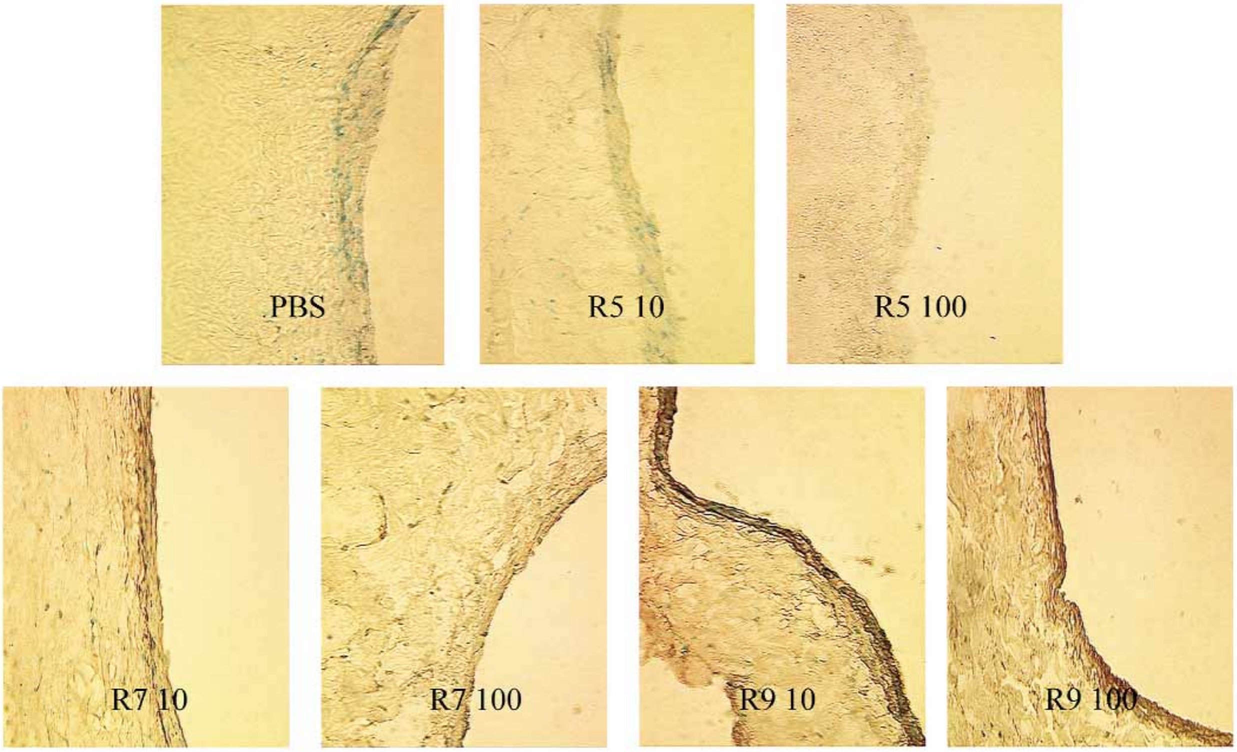


Fig 1. Representative figures of biotinylated L-arginine polymer and PBS-treated rabbit external jugular veins. Vessel lumina are on the right side of each photomicrograph. Nuclei are visible as *light green areas*, whereas *dark brown regions* signify areas of L-arginine polymer uptake. There is minimal biotin staining apparent in either PBS or R5 groups in contrast to the darkened nuclei and cytoplasm of R7 and R9 categories. (Original magnification, 400×.)

chemia followed by reperfusion after grafting, and traumatic endothelial cell loss caused by manipulation. These lead to the liberation of growth factors and cytokines that influence the migration and proliferation of vascular smooth muscle cells (VSMCs), as well as the deposition of extracellular matrix into the intimal compartment of the affected vessels.[3]

Nitric oxide (NO) is an important vasoactive molecule that limits neointimal hyperplasia by inhibiting VSMC proliferation and inducing cellular death by apoptosis.[4,5] It is a short-lived molecule that is formed by the activity of nitric oxide synthase (NOS) on its substrate, L-arginine. Although constitutive NOS is present in endothelial and neuronal cells, a third isoform, inducible NOS (iNOS), is expressed as a compensatory mechanism in vascular injury.[6] The synthesis of NO during these periods of elevated iNOS appears to be substrate limited,[7] and indeed, numerous investigators have used L-arginine administration to increase NO production during conditions of vascular stress.[8-10]

It has recently been noted that short polymers of L-arginine of 6 amino acids or greater in length are capable of translocating efficiently across cytoplasmic membranes of vascular cells.[11] Once located within the cell, these polymers can provide the L-arginine substrate for iNOS production of NO. The objective of this study was thus to determine whether L-arginine polymer treatment of vein grafts can enhance the production of vascular NO and inhibit the ultimate development of neointimal hyperplasia.

Methods

Peptide synthesis. Arginine polymers were synthesized on an Applied Biosystems 433 peptide synthesizer by using previously described solid-phase techniques and commercially available Fmoc amino acids, resins, and reagents (Applied Biosystems, Foster City, Calif, and Bachem, Torrance, Calif).[12] The following polymer sequences were used: L-arginine polymer (R) 5, NH_2-RRRRR-$CONH_2$; R7, NH_2-RRRRRRR-$CONH_2$; R9, NH_2-RRRRRRRRR-$CONH_2$; and phosphate-buffered saline (PBS) solution. Biotinylated polymers of each length were used in studies of translocation efficiency.

Animals. Male New Zealand White rabbits weighing between 2.5 and 3.5 kg were purchased from Krelek Farms (Stockton, Calif) and housed at the animal care facilities at the Department of Cardiothoracic Surgery, Stanford University Medical Center (Stanford, Calif). They were kept

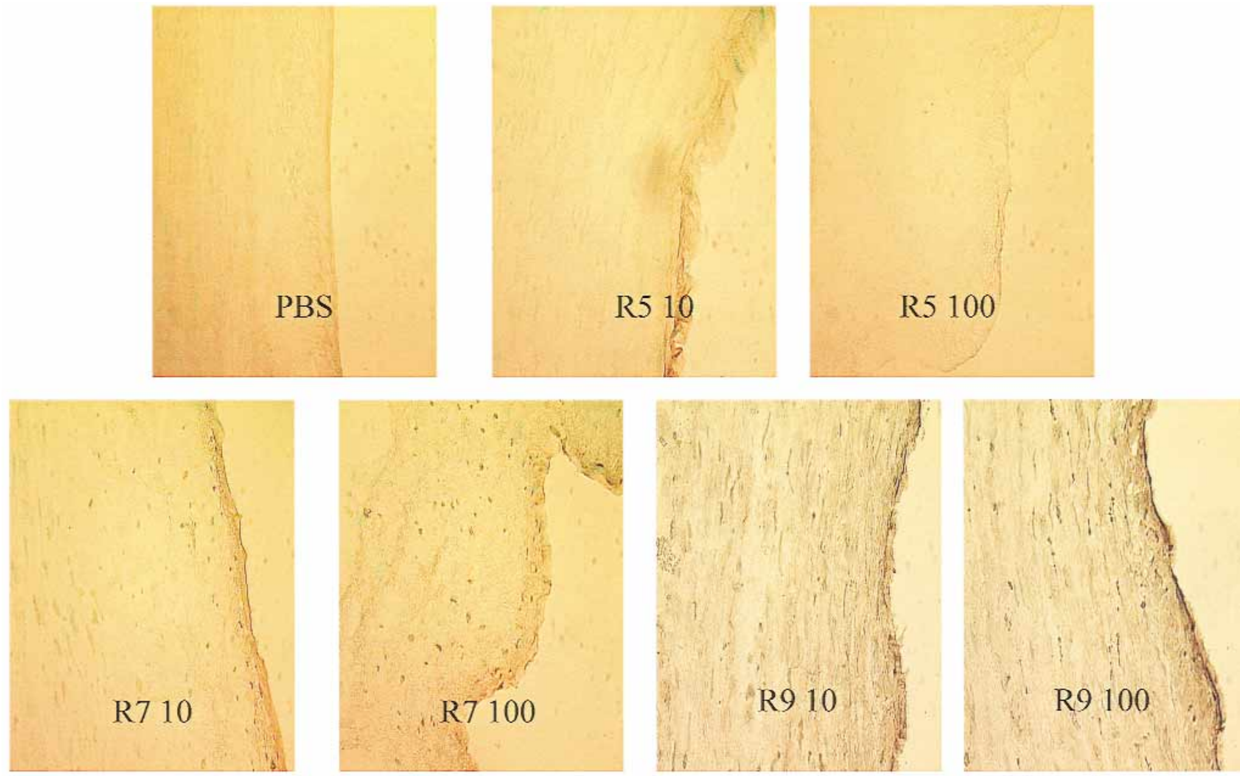


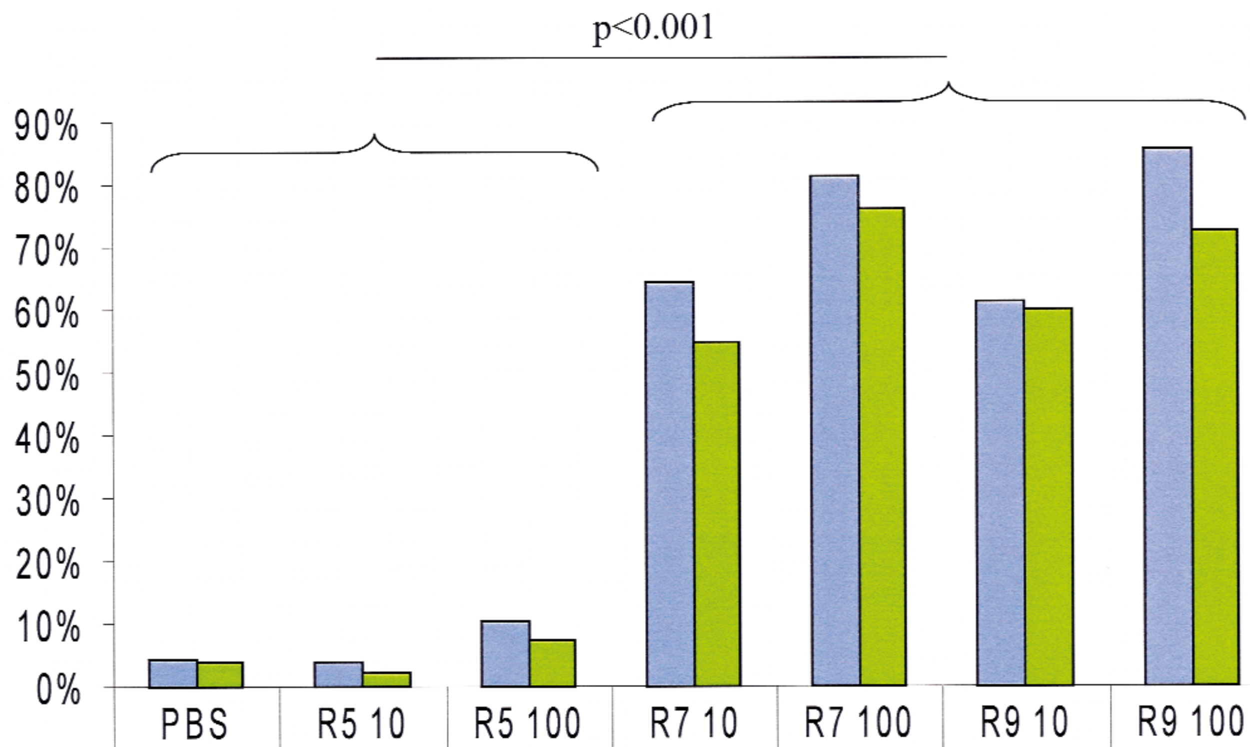
Fig 2. Representative figures of biotinylated L-arginine polymer and PBS-treated human saphenous veins. As is the case with rabbit veins, there appears to be little or no uptake of the polymers in either the PBS or R5 groups, whereas considerable expression of biotin is noted in the intima and media of vessels in both the R7 and R9 groups. (Original magnification, 400×.)

in rooms under normal temperature, humidity, and timed lighting conditions and were provided standard food and water ad libitum. Animals were treated in a humane manner in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press, revised 1996.

Surgical procedure. After anesthesia with intramuscular injection of 50 mg/kg ketamine and 5 mg/kg xylazine and mask inhalation of 2.0% isoflurane, the external jugular vein was isolated through a midline longitudinal neck incision. The vein segment was flushed of blood with normal saline solution and then instilled with either PBS, R5, R7, or R9 in solutions with concentrations of 10 or 100 µmol/L for 15 minutes (n = 6 per group). The segments were then reversed and anastomosed as an interposition graft in an end-to-side fashion to the contralateral carotid artery. The carotid artery segment between the proximal and distal anastomoses was then ligated and transected. Animals were killed after 28 days, and the vein grafts were perfusion fixed with 10% buffered formalin solution.

Translocating efficiency. To demonstrate the efficiency of arginine polymer penetration of vascular cells in vivo, the external jugular veins of New Zealand White rabbits were isolated as above and incubated for 15 minutes with either PBS or biotinylated R5, R7, or R9 in solutions of 10 or 100 µmol/L concentrations (n = 1 per group). These were then flushed with PBS solution before freezing in OCT compound and cutting into 5-µm sections. Frozen sections were fixed in acetone for 10 minutes at –20°C, washed in PBS solution for 3 separate 5-minute intervals, and incubated for 30 minutes with peroxidase suppressor (Pierce Chemical Co, Rockford, Ill) to block endogenous activity. Slides were then washed in PBS solution as above and incubated with 5 µg/mL horseradish peroxidase-conjugated streptavidin (Pierce Chemical Co) for 30 minutes. They were then washed and incubated for 60 seconds with the horseradish peroxidase substrate 3,3´-diaminobenzidine (Sigma Chemical Company, St Louis, Mo). Reactions were terminated by rinsing slides in distilled water before methyl green counterstaining and dehydration in ethanol.

Because of the relatively thin media in native rabbit veins, the same experiments were conducted in unused human saphenous vein segments obtained from patients undergoing coronary artery bypass grafting. The thicker vessel walls of the human vein segments allowed better appreciation of medial uptake. One vessel was treated per group, and 3 random high-power fields per cross section were analyzed for translocation of polymers. A quantitative measure of efficiency was calculated by dividing biotin-staining nuclei by total nuclei for both



	PBS	R5 10	R5 100	R7 10	R7 100	R9 10	R9 100
Intima	5%	4%	10%	64%	81%	61%	86%
Media	4%	2%	7%	55%	76%	60%	72%

Fig 3. Quantitative measure of translocation efficiencies on the basis of the number of biotin-staining nuclei *(brown)* divided by the number of total nuclei *(brown plus green)* in human saphenous vein samples. Each *bar* represents the average of 3 random high-power field readings of a cross-section of a single vessel. One hundred micromolar concentration appears to translocate with the highest efficiencies. Average translocation of the PBS and R5 group was significantly lower than that of R7 and R9 ($P < .001$, Student *t* test).

intimal and medial regions of these vessels, and an average of the 3 readings was taken. Statistical comparisons were made between a group comprised of PBS and R5 categories (n = 3), where little or no translocation was seen, and a group consisting of R7 and R9 categories (n = 4), where high translocation was present. The Medical Human Subjects Panel at Stanford University approved the use of human tissues in this study.

NO assay. Efficacy of L-arginine polymer therapy was assessed by means of in vitro assays for NO production. Separate rabbit external jugular veins were sectioned and treated with PBS solution or 100 µmol/L solutions of R5, R7, or R9 for 15 minutes (n = 6 per group). The vein sections were then incubated in Dulbecco's modified Eagle's medium (Gibco BRL, Gaithersburg, Md) containing both lipopolysaccharide (LPS; 100 µg/mL) and interferon (IFN) γ (200 U/mL; Sigma Chemical Co) to upregulate iNOS production. After 48 hours' incubation at 37°C, nitrite (NO_2^-) accumulation in the cell culture medium was directly quantified by the Griess reaction through a commercially available colorimetric assay (Cayman Chemical, Ann Arbor, Mich). Values were measured as micromolar concentrations and standardized to the weight of venous tissues in milligrams.

Vessel morphometry. Finally, the biologic effect of L-arginine polymer therapy was assessed by measuring the degree of neointimal hyperplasia in the 28-day treated and control vein grafts. The vein segments were placed in paraffin blocks, and the middle portion of each specimen was cut into 5-µm sections for staining with hematoxylin dye. A computerized morphometric analysis program (C-Imaging Systems, Cranberry Township, NJ) was used to measure intimal and medial areas of one section per graft in a blinded fashion. Neointimal hyperplasia is expressed as a ratio of these two measures (intima/media ratio [I/M]).

Statistical analysis. Values are expressed as means ± 1 SD. All comparisons were made by 1-way analysis of variance (ANOVA; SYSTAT Inc, Chicago, Ill). Unpaired Student *t* tests were used to identify statistical significance between groups, with the Bonferroni correction for multiple comparisons.

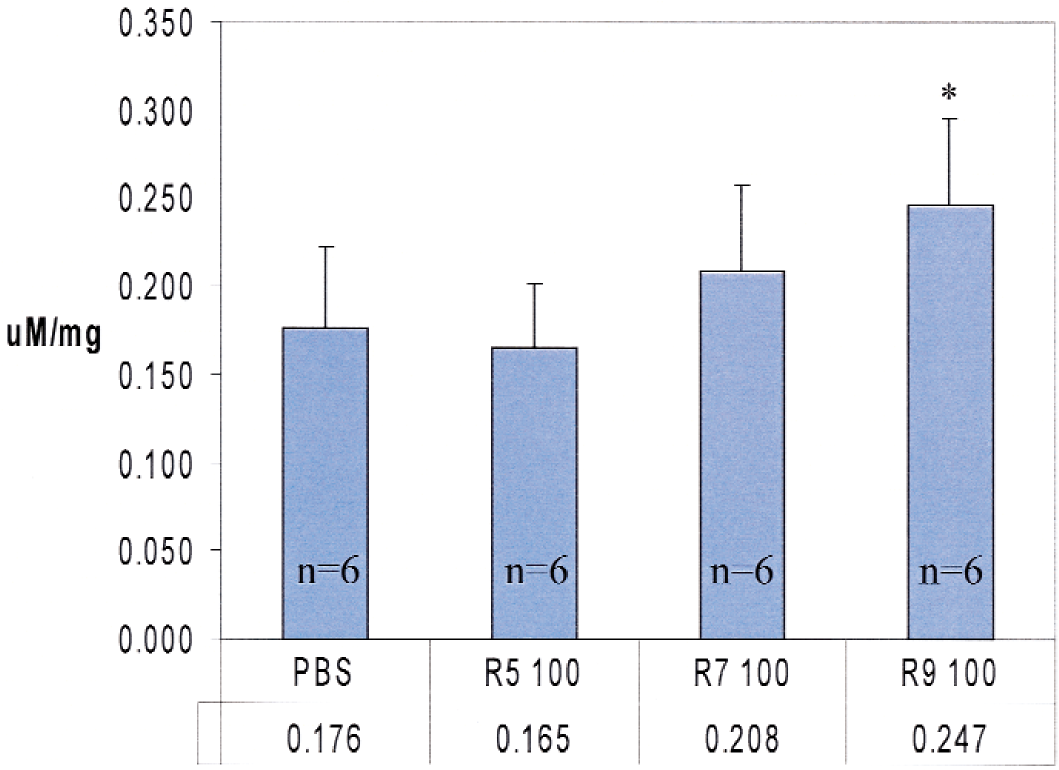


Fig 4. NO production as measured by nitrite (NO_2^-) levels. Pretreated venous segments are incubated in culture medium containing LPS (100 µg/mL) and IFN-γ (200 µg/mL) for 48 hours. Nitrite levels are measured directly from culture medium by means of Griess reaction and standardized to weights of venous segments. There is a length-dependent increase in nitrite levels with L-arginine polymer treatment. **P* = .033 versus R5 100 µmol/L and *P* = .085 versus PBS (Bonferonni-corrected value).

Results

Arginine polymer uptake efficiencies. Microscopic examination of rabbit jugular veins treated with biotinylated L-arginine polymers and PBS solution did reveal length-dependent uptake. Although R7- and R9-treated specimens exhibited evidence of vascular cell penetration to the level of the adventitia, neither the PBS nor R5 groups showed appreciable staining (Fig 1).

Translocation efficiencies were estimated in human saphenous vein segments because of the thicker medial layer allowing a more detailed analysis of uptake (Figs 2 and 3). R7 and R9 concentrations of 100 µmol/L resulted in the highest efficiencies at 81% and 86%, respectively. The intimal regions had greater uptake than their medial counterparts because of their direct exposure to treatment solutions. Neither PBS nor 100 µmol/L R5 groups had appreciable translocations. Average intimal translocation efficiencies were 6% ± 3% for the PBS and R5 groups versus 73% ± 12% for the R7 and R9 categories ($P < .001$, Student *t* test).

Measure of NO production. The effects of L-arginine polymers on venous production of NO were measured in vitro. Cytokine (IFN-γ) and endotoxin (LPS) stimulation were used to upregulate iNOS levels[13] in vein segments pretreated with either PBS solution or L-arginine polymers. Direct measurement of culture medium for the NO byproduct, nitrite (NO_2^-), revealed a length-dependent effect of the polymers at 48 hours with the R9 group having the highest levels ($P = .024$, ANOVA; Fig 4). There was also an incremental, although statistically insignificant, increase in NO in the R7-treated group when compared with either PBS or R5 solution.

Measure of neointimal hypertrophy. All animals survived until the time of harvest, at which point 88% of the grafts were noted to be patent by means of direct examination. There was no preponderance of early occlusion in any of the study groups. Although each vessel did have a degree of concentric neointimal hyperplasia (Fig 5, *A* and *B*), I/M ratios, as measured by means of computerized morphometric analysis, did demonstrate a protective effect of L-arginine polymers of sufficient length and concentration. One-way ANOVA yielded a highly significant difference among groups ($P < .001$). The I/M ratio in the PBS (0.91) and

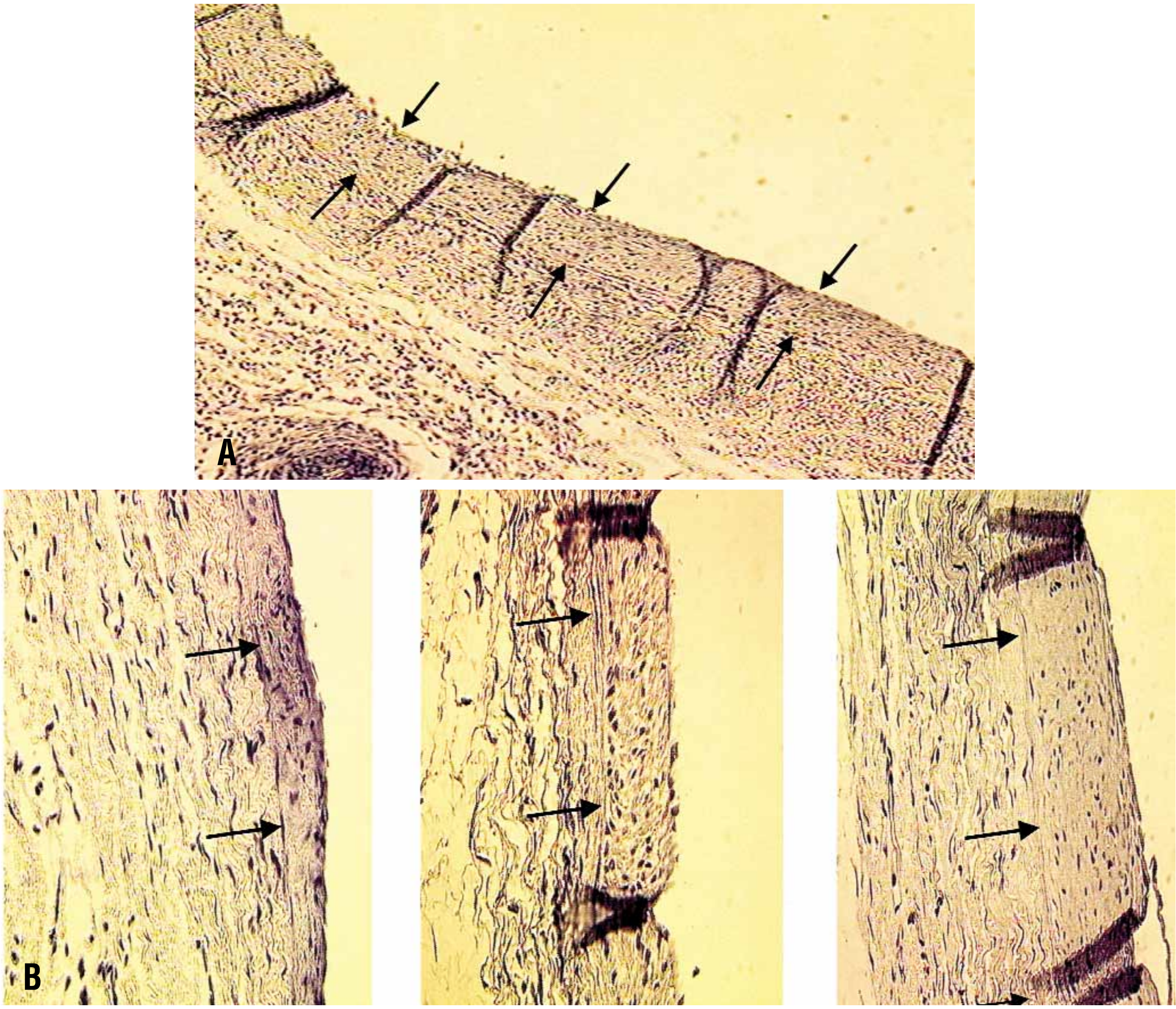


Fig 5. A, Low-magnification view (100×) of the concentric nature of neointimal hyperplasia visible at 28 days in vein grafts stained with hematoxylin. *Arrows* delineate the neointimal layer. **B**, Higher magnification view (400×) of low, medium, and high degrees of neointimal hyperplasia. *Arrows* depict the border between medial and neointimal layers.

both R5 groups (10 µmol/L R5, 0.92; 100 µmol/L R5, 0.92) were relatively consistent. A minimal reduction in neointimal hyperplasia was seen at the lowest concentration of R7 (I/M, 0.86); however, it was not statistically significant compared with that of the PBS group. Concentration appeared to play a more important role in efficacy of treatment in that 100 µmol/L R7 had a greater effect on I/M than 10 µmol/L of the R9 solution. Finally, the greatest effect on I/M was seen with the highest concentration and longest polymer length at 100 µmol/L R9. At 0.53, this group had an approximately 42% lower I/M ratio than the PBS or R5 control groups (Fig 6).

Discussion

Autologous vein remains one of the most useful conduits for revascularization of arterial occlusive disease.[9] Unfortunately, the development of neointimal hyperplasia in these vein grafts is nearly universal and significantly limits their long-term patencies. In this study we found that the ex vivo treatment of vein graft segments with L-arginine polymers upregulates their production of NO and limits the development of neointimal hyperplasia in a length- and dose-dependent manner.

Previous reports have described various techniques of manipulating NO production by vascular tissues. These include gene transfer of endothelial NOS[14] or

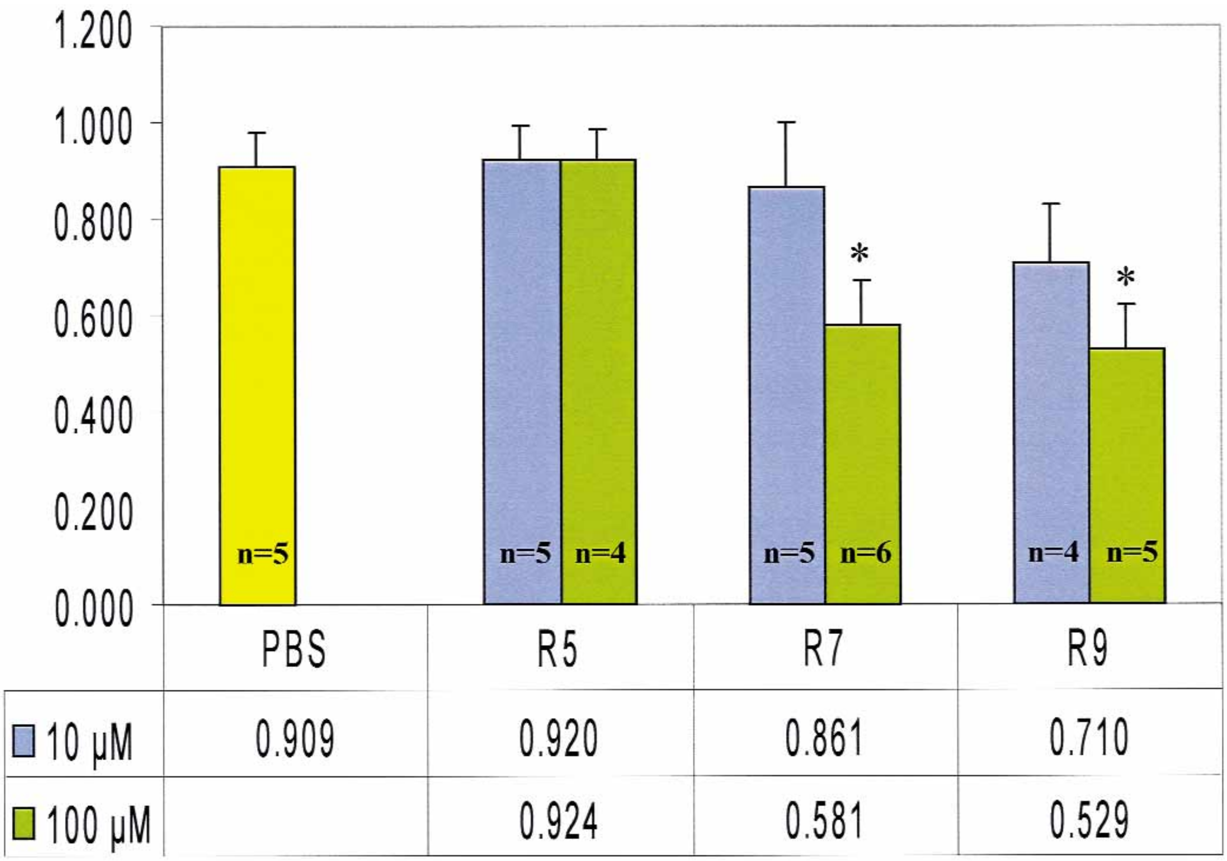


Fig 6. I/M ratio of treatment and control groups of 28-day-old vein interposition grafts. I/M is lowest in both 100 µmol/L concentrations of R7 and R9. *$P < .001$ verus PBS, R5 10 µmol/L, and R5 100 µmol/L (Bonferonni-corrected value).

iNOS,[15] the regulation of intracellular cyclic adenosine monophosphate in combination with proinflammatory cytokines,[16] and the dietary administration of the NOS substrate L-arginine.[17]

Dietary L-arginine reduces monocyte adhesion to vascular endothelium[18] and is thought to mediate its effects by increasing production of NO by these infiltrating cells.[19] The limitation of this mode of therapy, however, is the transient nature with which plasma L-arginine levels appear to be manipulated by dietary supplementation. Jeremy, McCarron, and Sullivan[19] found that despite continued oral administration of L-arginine, plasma levels remained elevated only up to 14 weeks. This was apparently caused by modification of L-arginine homeostasis either through increased metabolism by arginase[20] or enhanced urinary excretion.[21] The unsustainable elevations in plasma L-arginine content also correlated with a lack of a long-term benefit on vein graft endothelium-dependent vasodilatation in their model.

We have shown that single-dose therapy with L-arginine polymers of sufficient length and concentration inhibited the development of vein graft neointimal hyperplasia in an in vivo model. Sustained efficacy of other single-dose treatments has been noted previously. For example, ex vivo one-shot therapy with the NO donor S-nitroso-*N*-acetylpenicillamine successfully limited vein bypass neointimal formation in another study.[8] It appears that certain treatments centered around the time of vessel perturbation with bypass grafting are able to influence cell migration, matrix production, and other variables associated with vascular remodeling, such that there are long-term inhibitory effects on neointima formation.[22]

Histologic examination of the human vein grafts treated with biotinylated L-arginine polymers demonstrated that there is considerable uptake deep into the vessel wall at appropriate lengths and concentrations. Similar ability for membrane translocation was also seen with the Tat protein of the HIV-1 virus, which is rich in arginine sequences (residues 49-57; RKKRRQRRR).[23,24] Uemura and associates[25] found that this process of polyarginine uptake appears to occur in vitro through an energy-dependent mechanism (incubation with azide abolishes uptake) and independent of the normal amino acid transporter mechanisms. Incubation with excess concentrations of L-arginine monomers failed to have any appreciable effect on polymer uptake. If the polymers did gain access to the cell through the same transport mechanisms as the amino acid monomer, one might expect competitive inhibition at these levels.

Once within the cell, arginine polymers can supply the substrate for iNOS. This inducible form of NOS is upregulated within smooth muscle cells of vessels subjected to either endothelial injury[26] or proinflammatory cytokine stimulation.[27] Measurement of nitrite in our stimulated vein segments did demonstrate a length-dependent upregulation of the molecule that may be indicative of NO production by our treated venous grafts. These in vitro results were modest, however, and it is not known how long increases in NO would persist in treated arterialized vein segments.

The ultimate effect of L-arginine polymers on neointimal hyperplasia in vivo appears to be related to both the amount of substrate provided, as well as their ability to gain access to the cell. Neither concentration of R5 demonstrated any biologic effect on vein graft disease over PBS controls, and this is consistent with their lack of uptake. Interestingly, R7 at a concentration of 10 μmol/L, while having fairly efficient intracellular access, did not have any appreciable effect on the development of increased I/M ratio at 28 days. This suggests that although translocation is achieved, the ultimate effect on vein graft atherosclerosis also relies on the total substrate delivered to iNOS by the polymers. Indeed, the greatest effect on I/M is achieved with the maximum dose of 100 μmol/L and the longest length of R9, respectively.

NO is an extremely diverse molecule, and focusing only on its effects on VSMC proliferation may be overly simplistic. The upregulation of iNOS under conditions of endothelial damage may represent a unique response to injury protective mechanism for vessels subjected to various stresses. For example, NO-mediated vasodilatation and inhibition of platelet aggregation may be an attempt by an injured vessel to stave off early vein graft thrombosis.[28] Other investigators have demonstrated a protective role of exogenous NO delivery to hearts subjected to ischemia-reperfusion injury.[29,30] Future directions of study will look at the effects of L-arginine polymer therapy in these important areas and further delineate the unique roles of NO in vascular graft biology.

Finally, arginine polymers may have intrinsic benefits independent of their effects on NO synthesis. As the photomicrographs depicting the biotinylated polypeptides suggest, R7 and R9 are capable of efficiently translocating deep into vessel walls. By linking the polymers to specific therapeutic agents, they may thus have a distinct utility as a carrier protein for directed treatments of various cardiovascular diseases.

Conclusion

In conclusion, L-arginine polymers of sufficient length and concentration can efficiently translocate across membranes of vascular cells in ex vivo treated vein grafts. By providing the substrate for iNOS production of NO, these constructs represent a novel therapy for the inhibition of neointimal hyperplasia, which may currently limit the long-term patency of venous bypass grafts.

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Discussion

Dr Robbin Cohen *(Los Angeles, Calif).* Saphenous vein grafts continue to be the most common conduits used by cardiac surgeons for coronary artery bypass grafting. They are usually readily available, can be removed endoscopically with minimal morbidity, and have excellent and immediate flow characteristics. Unfortunately, despite a tendency to become functionally arterialized, a term I learned from Craig Miller, they tend to respond to being interposed into the coronary arterial circulation with neointimal hyperplasia, usually followed by accelerated atherosclerosis and premature occlusion.

You and your colleagues in Dr Robbins' laboratory are to be commended for tackling the huge problem of vein graft failure to make the results of our most commonly used conduit more long lasting. Your ideal scenario goes something like this: vein grafts that are rendered ischemic and damaged by their excision react by producing iNOS. An easily administered single-dose bath of L-arginine in the appropriate size polymers at the time of excision readily crosses the cell membranes of vascular cells and serves as a substrate for NOS, and the result is the production of NO, which fights off the proliferation of VSMCs, thereby inhibiting neointimal hyperplasia and presumably preventing the development of vein graft atherosclerosis. The study does provide potentially important data, which suggest that certain lengths of L-arginine polymers are taken up by vascular cells in a dose-dependent fashion. This is followed by an increase in NO production at 48 hours and a possible reduction in neointimal hypertrophy at 28 days, as measured with the I/M ratio. What the study does not do is justify your title. Although certainly a precursor, neointimal hyperplasia is not synonymous with atherosclerosis, nor was true atherosclerosis measured, observed, or really even mentioned in your article. Whereas your data warrant further investigation regarding the utility of bathing freshly harvested vein grafts in L-arginine, the assumption that it prevents atherosclerosis with the obvious benefits is premature. Still, your data are valuable, and I have 3 questions.

In your article there is about a 12% early occlusion rate of your vein grafts, which is not that dissimilar from what we see clinically. Did you look at those occluded grafts in a similar fashion to determine whether they produced increased NO, and what was their neointimal hyperplasia situation like?

Dr Kown. Thank you for your comments, Dr Cohen. Those 12% that were determined to be occluded were determined at the 28-day time point, well beyond the 48 hours that we used in our assays for NO production. It appears that these vessels were occluded because of early technical failure and not excessive neointimal hyperplasia in that histologic examination revealed only fibrous strands of scar tissue, with no evidence of any components of a vascular wall.

Dr Cohen. To perform your NO assay, you incubated vein sections in substances that are designed to upregulate iNOS production. Because your study makes ample L-arginine available to the vascular cell, to what extent is it actually dependent on that production of the iNOS? In other words, how reliable is that production? How much damage and ischemia has to occur for the production of NOS to occur, and how will that affect the production of NO down the line?

Dr Kown. There are 3 isoforms of NOS. One is the constitutive endothelial NOS, which is present in normal physiologic levels. If you did not have vessel perturbation associated with grafting, you would get a certain amount of NO produced by the vessel just by virtue of the fact that the endothelial NOS is present. This is easily discerned from how much production is created by a stressed vessel by adding a calcium ionophore because endogenous NOS is stimulated by calcium levels. When you do that, you see a slight incremental effect of NO produced by vein grafts; however, the predominant effect seen in our model when you have stressed vessels was from iNOS.

Dr Cohen. Finally, you chose as a single point in time, 28 days, to measure the I/M ratio and hence the degree of neointimal hyperplasia. Why not multiple time periods? Is this ratio on its way up or is it on its way down? Is this a long-lasting effect? Why did you not look at it a little bit more carefully?

Dr Kown. I think that is a good question. By studying our vein samples at 28 days for neointimal hyperplasia, we were trying to extrapolate how much actual vein graft disease occurs over the long term. The use of other models and time points would be a natural extension of these studies, but we believed that it was valuable to start with a snapshot of a single time point.

Dr Charles Brantigan *(Denver, Colo).* I would like to continue from where the previous discussant left off. Dr Cohen talked about providing arginine to eliminate the neointimal hyperplasia, and you talked about bathing the vein grafts in L-arginine or maybe flipping an on-and-off switch by presenting the bypass conduit with the L-arginine. How about a strategy that involves bathing the vein graft in arginine during the postoperative period by providing oral arginine to the animal or to the patient? There has been some evidence that suggests that giving L-arginine, which is available from a health food store, will in fact increase the levels of NO. We know that increasing the levels of NO decreases neointimal hyperplasia. Why not just provide something that is natural and free of side effects and thus provide some protection on a long-term basis? Have you investigated that strategy?

Dr Kown. We have seen from the literature that one way to upregulate NO is to provide dietary L-arginine. In one study with a hypercholesterolemic rabbit model, however, the authors found that for some reason, with oral administration of L-arginine, plasma levels were not maintained past 14 weeks. They attributed this to some sort of compensatory mechanism on arginine homeostasis by the body that led to increased urinary excretion and increased metabolism by arginase (which is the enzyme that will metabolize L-arginine). They thus found that they did not have a prolonged biologic effect past 14 weeks in that particular model.